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ORIGINAL PAPERS

KERATINASE ENZYME: VIRULENCE FACTOR IN PATHOGENITY OF DERMATOPHYTES

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Abstract. *Pathogenic dermatophytes are prevalent causes of a superficial cutaneous infection, which have the ability to invade keratinized structures such as skin, hairs and nails. Dermatophytes infection in the host involves three main steps: adherence to the host tissue, invading, and the development of a host response. In the first stage of infection, dermatophytes adhere to the surface of the keratinized tissue to reach the epidermis by using some factors that mediate adherence of dermatophytes. Various virulence factors are secreted from dermatophytes during the invading process in order to penetrate the host tissue, to obtain nutrients and survive. The aim of this study was to select keratin degrading fungi isolated from from clinical samples of patients with dermatophytosis. Application of soluble preparation of keratin (KS) of chicken feathers enables a preliminary evaluation of the growth of the fungi and screening of fungal isolates that possesed keratinolytic activity and keratinase enzyme. Five dermatophytes i.e. T.rubrum, T.mentagrophytes, M.gypseum, M.canis and C.tropicum were used in our study for keratinase activity. Out of five dermatophytes species studied, all tested fungal species showed a keratinase activity except one species i.e. M.canis. From our findings, these results provide a scientific validation that dermatophytes have keratinase enzyme activity and responsible for virulence factor in pathogenicity of dermatophytes.*

Keywords: Keratinase, virulence, dermatophytes, enzyme activity

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INTRODUCTION

Dermatophytes are keratinophilic fungi that belong to the genera *Trichophyton*, *Epidermophyton* and *Microsporum,* which exclusively infect the stratum corneum, nails or human hair. Groups of dermatophytes are divided into 3 groups according to their habitat: anthropophilic (human associated), zoophilic (animal associated) and

geophilic (soil habitat) [1]. Dermatophytes produce virulence factors such as keratinases and cellulase to penetrate stratum corneum of host tissues and produce disease. This is a common opportunistic pathogen that uses several kinds of virulence factors for infection. The keratinase is produced by dermatophytes [2] Dermatophytes are known to colonize the outer layer of the epidermis, which is rich in keratinous material such as skin, hair and nails. After dermatophytes adherence to keratinized tissue, the spores must germinate followed by penetration to the stratum corneum. The ability of dermatophytes to degrade keratin is considered a major virulence attribute [1]. During penetration, dermatophytes produce a variety of virulence factors for infection that include both enzymes and non-enzymes. Dermatophytes secrete a variety of virulence enzymes that have different substrate specificities such as protease, lipase and cellulase. Dermatophytes secrete many enzymes to obtain the nutrients to develop and survive. The macromolecules that are present in the host tissue are used as a source of carbon, nitrogen, phosphorus and sulphur for dermatophytes [3]. Moreover, it had been suggested that released enzymes from dermatophytes also act an antigen and induce various degrees of inflammation [4]. *Doratomyces microsporus* also produce keratinase enzyme and degrade skin epidermis *in vitro* under different experimental conditions [5]. At present, the knowledge of the factors that mediate adherence of dermatophytes is little known. Therefore, in the present study, an attempt was made to analyse the virulence enzymes such as keratinase produced by dermatophytes species.

MATERIALS AND METHODS

Collection of samples

Two hundred and sixty clinically suspected cases of dermatophytoses and other fungal diseases, attending the dermatology O.P.D. of E.S.I.C. & SMS Hospital, Jaipur during the one-year period were included in this study. A detailed clinical history regarding the patient's age, sex, occupation, socioeconomic status, symptoms, duration, climatic influence, family history, clothing and condition of the personal hygiene were recorded. Samples were collected from patients who were clinically suspected for dermatophytoses and other fungal diseases in sterile plastic bags. The infected areas or lesions were wiped with 70% alcohol to remove the dirt and other ointments. The specimens included skin scales, hair, hair roots, nail clippings and swabs. Clinical material was collected for microscopy and culture using standard mycological techniques.

Direct Microscopic Examination & Isolation of Fungi

Direct microscopic examination was undertaken in 10% potassium hydroxide (KOH) wet mount for the specimens of skin scales while 40% KOH was employed for hair and nail specimens (Emmons et al., 1977) Once the specimen was confirmed for the presence of fungal elements, the samples were streaked on the Sabouraud

Dextrose Agar (SDA) slants prepared with cycloheximide (50mg/L) and chloramphenicol (500mg/L) were used for culture. The culture tubes were incubated at 30°C and the culture growth was observed and the tubes were discarded only after six weeks in the absence of growth. The mycological identification was based on macroscopic and microscopic examination of the culture isolates. The macroscopic examination of dermatophytes was characterized by duration of growth, surface morphology and pigment production on the reverse.

Identification of isolated fungi

Isolated fungi were maintained on a Sabouraud's Dextrose Agar and identified by macroscopic and microscopic examination. For the macroscopic identification different selective media were used. Sabouraud's Dextrose Agar (SDA), Trichophyton Agar, and Sabouraud's Chloramphenicol Dextrose Agar were used for the growth and sporulation of different fungi. Positive cultures were examined both macroscopically (color of the surface and reverse, topography and texture) and microscopically (two types of conidia, small unicellular microconidia and larger septate macroconidia) for species identification, pigment production and further microscopy by Lactophenol Cotton Blue staining. Isolated dermatophytes were further identified from Institute of Microbial Technology (IMTECH), Chandigarh, India. Fungal strains selected for the study were *T.rubrum* **(MTCC 567)**, *T.mentagrophytes* **(MTCC 789),** *M.gypseum* **(MTCC 678)**, *M.canis* ***(MTCC 761)*** and *C.tropicum* **(MTCC 763)**

Keratinase Activity (Degradation of Keratin) by Dermatophytes

Keratinase activity or degradation of keratin substrate (chicken feathers) by keratinolytic fungi was studied according to Wawrzkiewicz *et al* method **(Wawrzkiewicz et al; 1991).**

Keratin media

The keratinolytic properties of the dermatophytes were examined on a solid mineral medium had the following composition:

$MgSO_4*H_2O$	-0.5 g
KH_2PO_4	-0.1 g
$FeSO_4*7H_2O$	-0.01 g
$ZnSO_4*7H_2O$	-0.005 g
Agar	-15 gm
Distilled water	-1000 ml
pH	-7.4

The media was supplemented with the keratin substrate as the sole sources of carbon and nitrogen. Chicken feathers or a soluble preparation of keratin protein of chicken feathers described as KS (Soluble keratin) according to Wawrzkiewicz *et al* (Wawrzkiewicz *et al.,*1991)

Preparation of keratin substrate

Soluble keratin was prepared from white chicken feathers. Native chicken feathers (10gm) in 500 ml of DMSO (Dimethyl sulfoxide) were heated at the temperature of about 100°C for two hours. Soluble protein was then precipitated from the solution by addition of cold acetone, using 2 volumes of acetone for 1 volume of the protein solution. The caseous precipitate of keratin protein was suspended in 0.1 M of phosphate buffer, pH 8. Afterwards, soluble keratin protein was added in the solid media at a concentration of 0.06%. Solid media were inoculated with micropipette, injecting 1ml of standard suspension into centre of the petriplates and petriplates were incubated at 37°C at an optimum pH 7.4 for keratinase activity. Control plates were prepared containing above medium without keratin substrate. Experiments were performed in triplicates and the presented results constitute the mean values. Formation of precipitate zones around the colonies indicate keratinase enzyme production by dermatophytes and zone was examined and measured.

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RESULTS AND DISCUSSION

Keratinase activity of dermatophytes and yeasts were studied under *in vitro* laboratory conditions on the solid mineral medium incorporated with soluble preparation of keratin protein (KS). Keratin substrate used for *in vitro* degradation

was chicken feathers. Five dermatophytes i.e. *T. rubrum, T. mentagrophytes, M. gypseum, M. canis* and *C. tropicum* were used in our study for keratinase activity. All fungal isolates used in our study, grew on the solid mineral medium containing 0.06% of keratin protein (KS) with 14 days of incubation. Out of five dermatophytes species studied, all tested fungal species showed a keratinase activity except one species i.e. *M.canis* **(Table 1)**.

Table: 1 Keratinase activity of Dermatophytes & Yeast species.

Fungal species	**Incubation Period**		
	3th	**10th**	**14th**
T. rubrum	-	20*/0**	20*/45**
T. mentagrophyts	20*/0**	20*/26**	22*/49**
M. gypseum	-	20*/34**	47*/65**
M. canis	-	-	20/0
C. tropicum	10*/20**	15*/25**	40*/60**

It was found that, the application of soluble preparation of keratin (KS) of chicken feathers enables a preliminary evaluation of the growth of the fungi and screening of fungal isolates that possessed keratinolytic activity and keratinase enzyme. Three replicates were prepared for each experiment and results constitute the mean values. *T. rubrum* showed keratinase activity on a solid mineral medium within incubation of 14 days. No growth of *T. rubrum* was observed on medium at third day of incubation. *T. rubrum* showed 20 mm diameter of fungal colony and no degradation on tenth day but on fourteenth day of incubation, *T. rubrum* colony (20 mm in diameter) was surrounded by a broad zone (45 mm in diameter) of degraded keratin, thus indicating an active secretion of keratin decomposing enzymes to the medium. *T. mentagrophytes* showed colony diameter of 20 mm at third day of incubation but clear zone of keratin degradation was not reported within 3 days but after on tenth day of incubation, colonies of (20 mm in diameter) surrounded by clear zone of (26 mm) diameter of degraded keratin was observed and on fourteenth day of incubation, large broad zone (about 49mm in diameter) of keratin degradation was found. Formation of clear zone around fungal colonies was reported on both tenth and fourteenth days of incubation. Zone of keratin degradation of fourteenth day of incubation was broader and larger than tenth day of incubation period. Growth of *M. gypseum* was not observed on third day of incubation. At tenth day of incubation,

both growth and clear zone of degradation was observed. Colony diameter of 20 mm surrounded by narrow clear zone of 34 mm was found and within fourteenth day of incubation, large broad zone of 65 mm was reported when compared with tenth day of incubation (34 mm in diameter). *M. canis* showed no growth on solid mineral medium at third day and tenth day of incubation. On fourteenth day of incubation, growth of 20 mm in diameter was reported but clear zone of keratin degradation was not observed around fungal colony. Thus, indicating an absence of active secretion of keratin decomposing enzymes into the medium. The excellent keratinolytic activity was observed in *C. tropicum* as shown in **Figure-1**. Within 3 days, these fungi grew on solid medium and also showed clear zone of 20 mm. Further increase in incubation period of 10 days, showed a higher zone of degradation (25 mm in diameter) than third day of incubation. On fourteen days of incubation, highest zone of keratin degradation of about 60 mm in diameter was reported. In the present study, increased keratinase enzyme activity for *M.gypseum, C.tropicum, T.mentagrophytes* and *T.rubrum* was found at optimum temperature i.e. 37±2°C and pH 7.4.

Figure: A

Figure: B

Figure: C

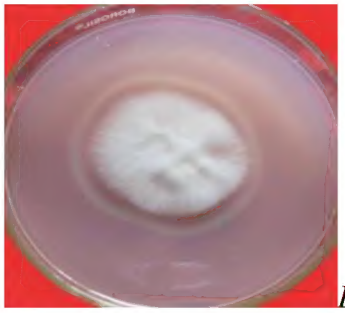
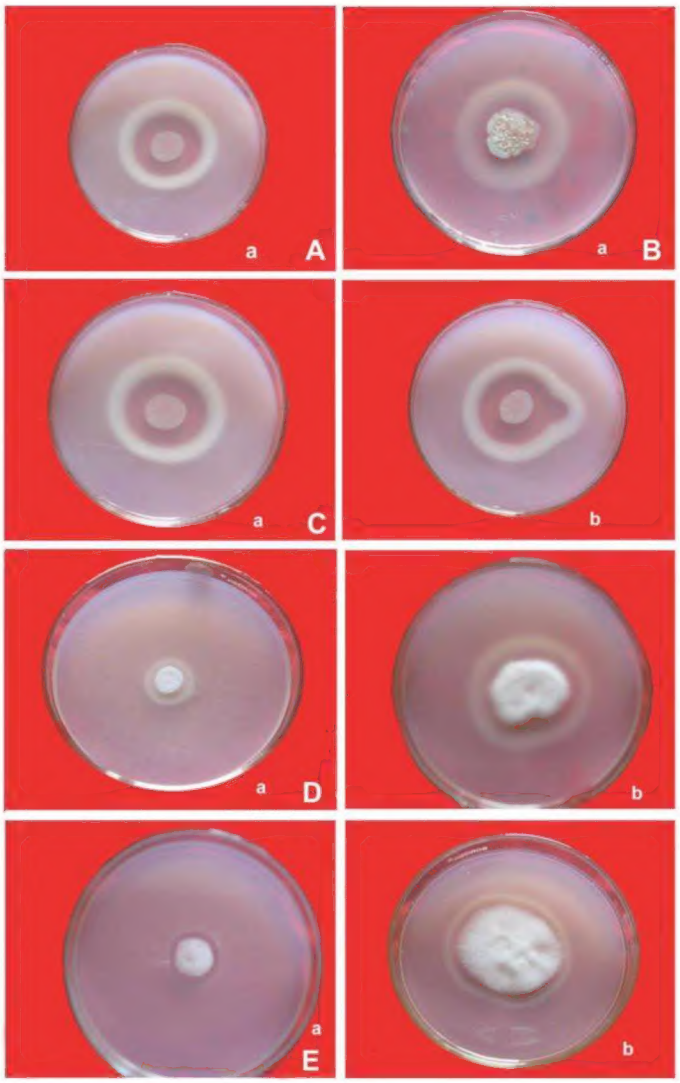


Figure: D

Figure: 1 A: *M.gypseum* **B:** *T.mentagrophytes* **C:** *C.tropicum* **D:** *T.rubrum*



DISCUSSION

In our study, degradation of keratin substrate (chicken feathers) by dermatophytes were studied. It was found that, *M. gypseum* (65 mm in diameter) showed highest keratinase activity followed by *C. tropicum* (60 mm), *T. mentagrophytes* (49 mm) and *T. rubrum* (45 mm). In our study, all the tested fungal isolates showed a diameter of clear zone of keratin degradation within range of 45-65 mm and possessed good keratinase activity except one species, *M. canis* which showed growth on solid mineral medium but clear zone of keratin degradation was not found by further increase of incubation period from 14 days to 21 days. This

reflected the fact that keratin is the main substrate for dermatophytes and therefore these fungi are called keratinophilic.

The examined species of dermatophytes are keratinase procedures and they are apparently capable of damaging the keratinized structure of the skin as previously reported [7]. The high keratinase activity of *M. gypseum* in comparison with other related fungi explain their ability to invade hair and cause keratin degradation. This was in agreement with work of earlier workers [8,9] which revealed that M. *gypseum* showed highest keratinase activity among examined dermatophytes.

The present experiments indicate a possibility of appearing modified keratin of chicken feathers / KS (Soluble keratin) as a very useful model for a preliminary estimation of keratinolytic activity of dermatophytes. This keratin, introduced as a source of carbon and nitrogen to the mineral agar medium, allows a quick selection of active strains. Native keratin contained in hairs or feathers did not constitute such a universal source of C and N for dermatophytes as the preparation of KS employed in our experiments. The few strains degrading keratin of guinea pig hair included strains of *T.verrucosum* and *T. mentagrophytes* of a wide infections spectrum [7]. In our studies, *T. mentagrophytes* also produced high keratinase activity (49 mm). Our results are also similar to Muhsin *et al.* [9] which revealed that the three tested varieties of *T. mentagrophytes* showed high keratinase activity. Sharma (2009) also reported that maximum keratinase (2.57± 0.028 unit/ml) was released from *T.mentagrophytes* when 35ºC temperature was provided [10]. In case of *M. canis,* in our results, keratinase activity was not detected. These results are in agreement with Wawrzkiewiez *et al.* [7] where *M. canis* was found negative for keratinase activity but disagree with the results of Muhsin *et al.* who reported (15 mm) zone of precipitation around fungal colony of *M. canis* [9]. *Chrysosporium tropicum* was also found in our study superior for keratinase production, forming zone of 60 mm after 14 days of incubation at 37°C. Our work was in agreement with El-Naghy *et al.* (1998) who reported that the *Chrysosporium georgiae* possessed high keratinase activity and completely degraded the added keratin after 9 days of incubation [11].

Moreira *et al.* (2007) investigated, degradation of keratinous materials by the plant pathogenic fungus *Myrothecium verrucaria* using poultry feathers as the only substrate [12]. According to da Gippo *et al.* (2009), the association of two residues poultry feather powder (PFP) plus cassava bagasse could be an excellent option as a cheap culture medium for the production of keratinase in submerged and solid-state cultures [13]. New feather degrading filamentous fungi was studied by Rodrigues Marcondes *et al.* (2008) and found that the highest keratinolytic activity were produced by *Alternaria tenuissima* after 4-6 days of cultivation in submerged conditions followed by *Acremonium hyalinulum, Curvularia branchyspora, Beauveria bassiana* [14]. The results of this work contribute to showed that keratinolytic activity was relatively widespread among common filamentous fungi and may have an important role in keratin decomposition. According to Gupta and

Ramnani (2006), microbial keratinases have become biotechnologically important since they target the hydrolysis of highly rigid, strongly cross-linked structural polypeptide "keratin" recalcitrant to the commonly known proteolytic enzymes trypsin, pepsin and papain [15]. These enzymes are largely produced in the presence of keratinous substances in the form of hair, feather, wool, nail, horn etc. during their degradation. Raju *et al.* (2007), in their studies, clearly indicates the presence of enzyme keratinase in the dermatophyte *Microsporum gypseum* and found that maximum biomass and keratinase activity was observed at pH 8 and at 35°C [16]. Muhsin *et al.* (1997) also found in their studies that *T. rubrum* and *C. albicans* also showed good keratinase activity which was coincides with the results of our present work [9]. *T. rubrum* produced clear zone of 45 mm in our studies. Difference in the properties of keratinases in particular strains of dermatophytes have been noted by Takiuchi *et al.* (1982) [17].

CONCLUSIONS

Superficial dermatophytosis is a common fungal infection in humans. In pathogenicity, dermatophytic adhesion is begins with the use of mediate adherence factors. During penetration, dermatophytes secrete several kinds of virulence factors that are key factors in the invasion and utilization of the stratum corneum of the host. Therefore, an understanding of the specific virulence factors involved in pathogenicity of dermatophytes would assist in the development of new therapeutic approaches. These results concluded that dermatophytes were capable of producing keratinase enzyme as a virulence factor in pathogenicity of dermatophytes.

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